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TITLE: *Tbx20* function in Proepicardial organ (PEO) & epicardium derived cell differentiation and *Asporin /PLAP-1* function in aortic valve calcification

Summary of work

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Enrolment No.: SC/DOC/13-14/35

Registration No.: 14RS18210033

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SYNOPSIS (5000WORDS)

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1. Introduction

Heart development or cardiogenesis is a very complex process in vertebrates in utero. Interestingly, the heart is the first pulsatile functional organ developed during embryogenesis. In a developing embryo, the heart appears as a tubular structure and it is derived from precardiac mesoderm (Asli, Xaymardan, and Harvey 2014). The first stage of cardiac morphogenesis involves cyto-differentiation of mesoderm forming two distinct tissue layers. One is contractile myocardium and other is inner endocardium. Thereafter, the third cell layer, epicardium is formed covering the whole heart (Wijk et al. 2010). Errors in these developmental stages affect cardiogenesis and result in different cardiac malformations and abnormalities. Worldwide, a wide spectrum of heart diseases is appearing in many developing countries including India. Current statistics suggest that around 30-35 million heart patients are being treated and around two lakhs surgeries are being performed every year (Go et al. 2013; Update and Control 2018). Among all these heart-associated diseases, two serious concerns are heart attack or myocardial infarction (MI) and heart valve calcification. Research on regenerative therapies to replenish lost cardiomyocytes (CMs) after MI, is essential to enrich the cardiovascular field so far. Similarly, molecular hierarchies, which are associated with valvular calcification, should also be investigated in greater details. Thus, these targeted objectives will provide us significant impacts in cardiac repair/regenerative strategies in near future.

In vertebrate cardiogenesis, the outer epicardial layer is derived from the extra-cardiac pro-epicardial organ (PEO). PEO is an early-developed organ, which has a typical transient grape like structure. It is situated at the venous pole in between atrio-ventricular (AV) junction of the embryonic heart. Embryonic PEO-derived cells start to migrate forming an envelope of the whole heart, called as epicardium (Pérez-Pomares et al. 2001). Epicardium-derived cells (EPDCs) undergo epithelial to mesenchymal transition (EMT) and differentiate into various cardiac lineages specific cell types. These cell types are smooth muscle cells, fibroblasts, endothelial cells and also cardiomyocytes (Gittenberger-de Groot et al. 1998; Wessels and Pérez-Pomares 2004; Austin et al. 2008). Most of the previous reports have been centered on non-cardiomyocyte (smooth muscle cells, fibroblasts and endothelial cells) lineage specification from EPDCs. Comparatively less number of reports have been published about myocyte (cardiomyocyte, CM) lineage differentiation potential of EPDCs. Interestingly, there

are several crosstalks regarding CM differentiation from EPDCs. Few groups have suggested that EPDCs have the potential to be differentiated into CMs (Kruithof et al. 2006; C. L. Cai et al. 2008) whether other groups have revealed that EPDCs do not have the contribution in CM specification (Duan et al. 2012). So, here more studies are needed to discover the EPDC potential in CM lineage differentiation. In fact, very limited reports have discussed about PEO-derived cells and its independent potential in cardiac morphogenesis (Buermans et al. 2010). T-box transcription factor gene, *Tbx20* is known to express markedly in different cardiac cell types including ventricular myocardium and epicardium (Iio et al. 2001; Yamagishi et al. 2004). In addition, previous reports have suggested that *Tbx20* knockout mice and human have shown defects in heart development and chamber formation (Kirk et al. 2007; C.-L. Cai 2005). *Tbx20* plays multiple roles in embryonic cardiogenesis at early stages and promotes cardiomyocyte cell proliferation (Singh 2005; Shen et al. 2011; Shelton and Yutzey 2007; Chakraborty, Sengupta, and Yutzey 2013). Previous unpublished data have stated that in the adult murine heart, there is an increased expression of *Tbx20* in injured myocardium and epicardium after 4 weeks of MI. There are several signaling pathways, including Wnt/β-catenin signaling and BMP/FGF signaling, which are extensively involved directly in epicardial differentiation mechanisms (Nusse 2008; Kalani et al. 2008; Blauwkamp et al. 2012; Beederman et al. 2013). Together, investigation of *Tbx20* function in lineage differentiation from embryonic PEO-derived cells and EPDCs and associated signaling pathways, might be worth exploring in this area. Therefore, the first aim of this research is to study different lineage specific gene expression patterns of PEO-derived cells and EPDCs in explants. Also, determination of *Tbx20* function in cardiomyocyte specification from PEO-derived cells and EPDCs, has been included in this study.

On the other hand, it had been studied that embryonic mesenchymal cells (MSCs) of the out flow tract (OFT) cushions get differentiated into mature aortic valvular cusps in adult (Lincoln, Alfieri, and Yutzey 2004) during cardiogenesis. Certain developmental events include proliferative, migratory and undifferentiated mesenchymal cells of endocardial cushion, which give rise to mature aortic valves with dynamic extracellular matrix (ECM) (Chakraborty et al. 2008; Rutkovskiy et al. 2017). Then mature aortic valve leaflets are stratified into elastin-rich atrialis/ventricularis, proteoglycan-rich spongiosa and highly organized collagen fiber-rich fibrosa. Interestingly, adult aortic valve calcification characterizes thickening of valvular cusps due to matrix mineralization and calcification and causes calcific aortic valve diseases (CAVDs). As a result proper cardiac function gets

compromised due to narrowing of the path of unidirectional blood flow. Clinical studies suggest that biological phenomenon of valvular mineralization and calcification is actually similar with osteogenesis (Rajamannan et al. 2003; Lincoln, Lange, and Yutzey 2006). Because shared gene regulations and signaling pathways are identified between valve and bone development. Asporin or PLAP1 (Periodontal ligament associated protein 1) is a small leucine rich proteoglycan which has a key role in the negative regulation of bone cell calcification (Yamada et al. 2007). It is reported that *Asporin* or PLAP1 mRNA is strongly expressed in adult aortic valve tissue (Chakraborty et al. 2008). Other reports have postulated that Asporin regulates via BMP2-pSmad1/5/8 signaling pathway and inhibits calcification. It deactivates BMP2-pSmad1/5/8 signaling through competitive binding with BMP receptor (Tomoeda et al. 2008; Song et al. 2015). On the other hand, Wnt/β-Catenin signaling is involved in bone cell differentiation. Previous reports have shown that this signaling gets activated in bone cell lineage differentiation and promotes osteogenesis (Alfieri et al. 2010; Gu et al. 2014). No such study has been focused on Asporin function and associated signaling mechanisms to regulate aortic valve mineralization and calcification before. Therefore, the second aim of this research is to study Asporin function in aortic valve mineralization and calcification and has been included in this study.

Despite several reports on EPDCs differentiation and aortic valve calcification, many other mechanisms have been poorly understood and yet to be identified (Sinha 2004; Grigoryan et al. 2008; Russell et al. 2011; Buermans et al. 2010; Xia et al. 2017; Gu et al. 2014; Yamada et al. 2007; Hampson et al. 2013; Acharya et al. 2011; Cheek et al. 2012). This report has been stating following major objectives, i) Selective gene expressions including *Tbx20* have been analyzed in cardiomyocyte lineage specification from PEO-derived cells and EPDCs in avian explants, ii) Role of Wnt/β-Catenin signaling in PEO-derived cells and EPDCs potential to form CMs in avian explants, iii) Selective gene expressions and signaling pathway marker expressions of avian adult aortic valvular interstitial cells (AVICs) maintained in osteogenic and control media including *Asporin*, iv) Effects of Wnt/β-Catenin signaling manipulation and Asporin over-expression on AVICs mineralization.

Overall, these new findings will be able to reveal new insights into epicardial biology and novel therapeutics in aortic valve calcification process with a better understanding of the normal cardiac developmental process.

2. Objective of the study

- **Aim1: Studies on avian PEO and epicardium derived cell differentiation potential in explant culture.**

  A. Differential gene expression pattern of several cardiac lineage specific differentiation markers in avian PEO derived cells versus epicardium.

  B. Determination of *Tbx20* function in myocardial cell lineage specification from PEO derived cells versus epicardial cells by manipulation of **i)** Wnt/ β-catenin and **ii)** BMP2-pSmad /1/5/8 signaling.

- **Aim2: Asporin function in aortic valve mineralization and calcification.**

  A. To determine the role of Asporin as a negative regulator of aortic heart valve calcification.

  B. To determine the functional interrelation between Asporin and Bmp2-pSmad1/5/8 signaling in aortic valvular interstitial cell mineralization.

3. Methodology

3.1. Sample collection:

Fertilized incubated (38°C) avian eggs were collected from local hatchery (State poultry farm, Tollygunge, Kolkata, West Bengal) at embryonic day 3, 4 and 5 (E: 3, 4 & 5). The specific avian breed used was *Gallus gallus domesticus* (Rhode Island Red and Black Australorp) (Polley et al. 2017). All the fertilized eggs were procured in accordance with the University purchase guidelines. Adult avian hearts were obtained from local hatchery (State poultry farm, Tollygunge, Kolkata, West Bengal).

3.2. Establishment of primary avian explant cultures:

A. *Avian PEO explant culture:*

After collecting fertilized incubated avian eggs from the hatchery, embryos were isolated. Then proepicardial organ or PEOs were dissected out from atrio-ventricular (AV) junction in whole embryonic heart at E: 4. Dissected PEOs were seeded onto a 0.01% collagen (CollagenI Rat tail, 3mg/ml, A10483-01) coated two-well chamber slides (SPL,30122 or Eppendorf, 0030742052) or plates with 1ml complete media [M199 (Invitrogen,11150-059) and supplemented with 10% fetal bovine serum (Invitrogen,10270), 1% penicillin/streptomycin (Pen-Strep) (Invitrogen,15140122)]. After 24hrs, the PEO explant cultures were used to perform all the experiments.

B. *Avian embryonic epicardial explant (eEpi) culture:*

Embryonic epicardial (eEpi) explant cultures were established by isolating whole hearts from avian embryos at E: 5. Whole hearts were cultured in 0.01% collagen coated plates containing M199 complete media and allowed to attach at the bottom for overnight. Then the hearts were removed leaving the formed eEpi monolayers. These eEpi monolayer cultures were consequently used for further experimentations.

C. *Adult avian aortic valvular interstitial cell culture:*

Primary aortic valve interstitial cells (AVICs) were established from aortic valvular cusps of adult avian hearts following previously described protocol (Alfieri et al. 2010). Briefly, adult valvular leaflets from aortic base region were dissected out. Next, these cusps were explanted and subjected to treatment with collagenaseII (Sigma 234153) M199 medium (Invitrogen, 11150-059) supplemented with 1% penicillin/streptomycin (Pen-Strep) (Invitrogen, 15140122) at 37∘C, 4×7mins in a shaking water bath. Pulled supernatants from 10-12 valves were centrifuged at 800 rpm for 5 minutes and the pellet was resuspended in 3ml complete M199 culture medium. Then the final cell suspension was sieved through nylon mesh and plated onto 0.01% collagen coated plates containing complete M199 media followed by 2-5 days of culture. AVICs explant cultures were established without any indicated contaminations and these were used to perform all the experiments.

D. *Osteogenic Induction:*

To study osteogenic gene induction, cultured cells were treated with osteogenic medium containing complete M199 medium, β-glycerophosphate (10mM), L-ascorbic acid (10nM), Dexomethasone (0.1μM) as described before (ref). Osteogenic (OS) medium was added after 3 days of AVICs culture and kept in treatment for a maximum of 3days (72hrs) in healthy condition.

3.3. Selective marker gene expression analyses:

A. *Reverse transcriptase PCR:*

PEO and Epicardium specific markers *(Wt1, Tbx18),* T-box transcription factor, *Tbx20,* cardiomyocyte specific markers (*Mefc2c, Gata4*), Osteogenic Markers (*Osteopontin, Runx2*), Wnt/β-Catenin signaling pathway markers *β-Catenin* have been analysed. The details of primer sequences, annealing temperature and produce size are provided in Table I.

Total RNA was isolated from dissected and pulled twelve PEO tissue samples of E: 3 or E: 4 avian embryos per experimental group using 200μl of Trizol (Invitrogen, 15596026). 1μg of total RNA was then reverse transcribed to cDNA using Bio-rad kit (iScript^{TM} Reverse transcription supermix, 170-884) according to manufacturer's supplied protocol. Total RNA was also isolated from PEO (E: 4) and eEpi (E: 5) explants using 200μl of Trizol. Then cDNA was synthesized, taking 1μg of RNA by RT-supermix. Semi qRT- PCR was performed using DNA taq polymerase (Bioline, BIOTAQ DNA polymerase BIO-21040). These PCR were performed for 35 cycles using 20pmol of the following primer pairs (IDT).

B. *Quantitative real time PCR (qRT-PCR):*

Quantitative gene expression analyses have been performed using above selective markers. qRT-PCR was performed for further validation of semi qRT-PCR in 10 μl of total volume using Bio-Rad Real Time PCR kit (SSO fast Eva green supermix, 172-52 03AP) according to previously standardized protocol (Alfieri et al. 2010). Gene expression levels were determined by Cq value obtained from Bio-Rad CFX manager software normalized to *β-Actin* expression. qRT-PCR results for each gene represents the data generated from at least three biological and technical experiments (n=3). For both PEO tissues and explant cultures, gene expression was evaluated as increased or decreased fold change calculated by

experimental Cq values. Statistical significance was calculated for observed differences by student's t-test and considered significant for *p* values (≤0.05).

3.4. Immunostaining:

PEO and eEpi explant cultures were fixed in paraformaldehyde (Affymetrix, 19943). Then immunostaining was performed according to manufacturer's supplied protocol. The following antibodies were used for immunofluorescence (IF): Mf20 (1:200 dilution, DSHB, ID-AB_2147781), Ki67 (1:400 dilution, ab15580). A mouse polyclonal secondary antibody (Goat anti-Mouse IgG H&L, Alexafluor 488, ab 150113) directed against Mf20. Likewise a rabbit polyclonal secondary antibody (Goat anti-Rabbit IgG H&L, Alexafluor 488, ab 150077) was applied against Ki67 primary antibody at 1:1000 dilutions in blocking solution. It was used at 1:1000 dilutions in blocking solution. All primary antibody incubations were done for overnight at 4°C. Nuclei were stained with DAPI (abcam, ab104139) in fluoroshield mounting medium.

AVICs explant cultures were fixed in paraformaldehyde. Then immunostaining was performed according to manufacturer's supplied protocol. The following antibodies are used for immunofluorescence (IF): Asporin (1:200 dilution, DSHB, ID-AB_2147781) and α-Sma (1:400 dilution, abcam, ab5694). A rabbit polyclonal secondary antibody (Goat anti-Rabbit IgG H&L, Alexafluor 488, ab 150077) was applied against Asporin primary antibody. All primary antibody incubations were done for overnight at 4°C. For all immunostaing nuclei were stained by DAPI (SRL, 18668). (n =3)

3.5. Assessment of matrix mineralization and calcification by Alizarin Red S staining:

For qualitative and quantitative analyses of mineralization of adult AVICs, cells were harvested and cultured according to a standardized protocol. Then, cultured cells were taken and carried out with OS treatment followed by other treatments. After that cells were fixed with 4% paraformaldehyde in PBS for 45 minutes at 4°C. For qualitative analyses, 2% Alizarin Red S (Sigma) was used and for quantitative analyses 40mM Alizarin Red S was prepared (Yu et al. 2017).

After cell fixation, cells were stained with freshly prepared 2% Alizarin for 30minutes. Then cell plates were washed with distilled water (dH_2O) and dried. Digital bright field images

were taken on LASX software (Leica). On the other hand, fixed cells were stained freshly prepared 40mM Alizarin Red S for 30-40mins with gentle shaking at room temperature. Then following standardized protocol (Acharya et al. 2011), OD values were taken of the control and OS induced samples using Gene5 software (Biotek, microplate reader). Then final quantitative difference was calculated with proper statistical analyses. (n =3)

3.6. In vitro activator and inhibitor assays in avian explants:

A. *Activator assays:*

For Wnt signaling activation, lithium chloride (LiCl) or recombinant Wnt3a protein is used as described previously by others (Sinha 2004; Alfieri et al. 2010). After successful establishment of eEpi culture, cells were treated with 15mM Lithium Chloride (LiCl) (Sigma, 203637) or 150ng/ml recombinant Wnt3a protein (Abcam, ab81484) supplemented with M199+Pen-strep media as described before. After 48 hrs of culture of eEpi culture control cells were kept in M199+Pen-strep and experimental plates were kept in LiCl or Wnt3a supplemented M199+Pen-strep medium for 24 hrs. After indicated hrs, control and treated plates were carried forward for detailed gene expression analyses and immunostaining.
AVICs were treated with recombinant human Wnt3A (rhWnt3A, 150ng/ml) protein after 2days of culture followed by 3days of osteogenic induction.

B. *Inhibitor assay:*

eEpi cultures were established as stated before and treated with Xav939 (Abcam, ab120897) for Wnt signaling inhibition assay. After 48hrs of eEpi culture, 5 μM of Xav939 were supplemented with M199+Pen-Strep and treated to the eEpi cells for 7hrs as described previously (Chakraborty, Sengupta, and Yutzey 2013). After indicated hrs, control and treated plates were carried forward for detailed gene expression analyses. AVICs were treated with Xav939 (5 μM) after 2days of culture followed by 3days of osteogenic induction.

3.7. Gain of Function (GOF) analyses of Asporin:

Adult avian AVICs were cultured following protocol and osteogenesis is induced. AVICs were treated with recombinant human Asporin protein (150ng/ml; Abcam132382) in the

presence of OS media. After indicated hrs, control and treated plates were carried forward for detailed gene expression analyses and further experiments.

3.8. Coomassie blue staining

For morphological study, the isolated proepicardial (PEO) and embryonic epicardial (eEpi) cells were stained with coomassie brilliant blue after 48 hrs of culture. Cultured cells were washed once with 1X PBS and given coomassie brilliant blue (0.1%) solution for 30 mins. After that coomassie blue stained slides were air-dried for 1-2 hrs and bright field images were captured using inverted microscope (Zeiss).

3.9. Bright-field and fluorescence microscopy

PEO and eEpi explant cultures were also being imaged at bright-field using inverted microscope (Zeiss). AVICs cultures were also being imaged at bright field using inverted microscope (Zeiss). For immunostained slides, fluorescence was detected using upright and inverted fluorescent microscopes (Zeiss lab, flu microscope and Leica fluorescence microscope) and images were captured using zeiss software: ISC capture and Leica software: LAS X.

3.10 Statistical Analyses

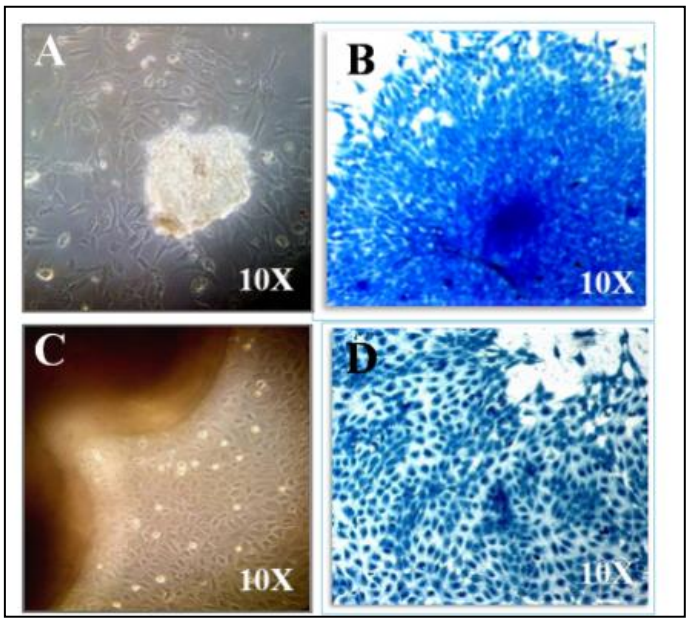
Student's t-test, specifically one sided two-tailed was used to detect the statistical significance ($p \leq 0.05$) and to derive all the fold changes in gene expression assays. All experiments were carried out at least in both biological and technical triplicates. In addition, all auto calculated values were taken and after generating the mean values in each set of experiments, control value was set at 1. Then fold changes of respective experiments, were calculated with respect to the control value by unitary method. Also for Mf20 and Ki67 experiments, a minimum of 500 cells was counted in each set of experiments (n=3). All the cell count values were taken and after generating the percentages in each set of experiment, control value was set at 1. Then fold changes of respective experimental sets, were calculated with respect to the control as mentioned previously.

Overall, all the statistical analyses have been reported with standard error of mean (SEM) subjected to student's t-test ($p \leq 0.05$).

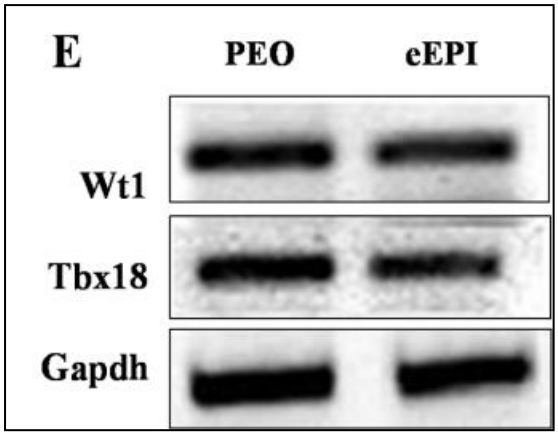
4.Results

The following results are generated from proposed Aim 1

4.1. Successful establishment of primary avian embryonic Proepicardial organ (PEO) and embryonic (eEpi) Epicardial explants

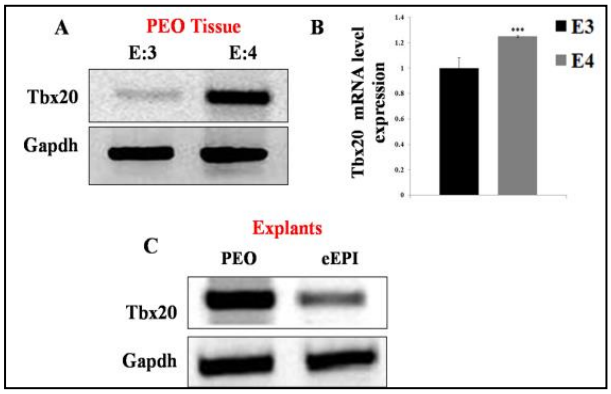


Both primary avian PEO and eEpi explant cultures are established from E: 4 and E: 5 embryos respectively. Bright-field image is shown in figure A indicating PEO monolayer cells migrated from explanted PEO tissue. Next, coomassie brilliant blue stain is used to demonstrate overall cellular morphology of cells in cultures. Also, figure B depicts the diverse types of cardiac cell morphologies. In contrast, as shown in figures C and D, typical cobblestone like epicardial cell morphology is detected by both bright-field and coomassie brilliant blue stained images.



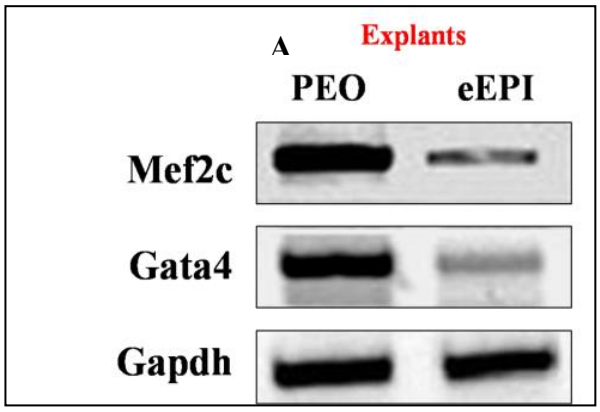
After successful establishment of both primary PEO and eEpi explant cultures, PEO and eEpi marker gene expressions have been checked by semi quantitative reverse transcriptase PCR (semi qRT- PCR). As expected, PEO and eEpi specific *Wt1* and *Tbx18* have been detected with comparable expression in both explants (figure E). *Gapdh* is used as loading control. Overall, both PEO and eEpi explant cultures are successfully established and considered for experiments.

4.2. *Tbx20* expression in avian embryonic PEO and Epicardial cells



Tbx20 is known to express in multiple cardiac cell lineages during early stages of cardiogenesis. Therefore, to determine if *Tbx20* expresses differentially during early and late stages of PEO development, a comparable gene expression pattern has been studied on isolated RNA from E: 3 & E: 4 PEO tissues. The expression of *Tbx20* has been analyzed by semi qRT-PCR and quantitative real time PCR (qRT-PCR) after PEO specific cDNA synthesis. In semi qRT-PCR data, *Tbx20* has shown increased band intensity at E:4 (figure A) and in qRT-PCR, it has been significantly upregulated by 1.25 fold (figure B) as compared to E: 3. Moreover, semi qRT-PCR data has suggested that *Tbx20* band intensity is also markedly increased in PEO-derived cells compared to eEpi cells (figure C) in culture. *Gapdh* is used as loading control. Thus, *Tbx20* markedly expresses in embryonic PEO and epicardium.

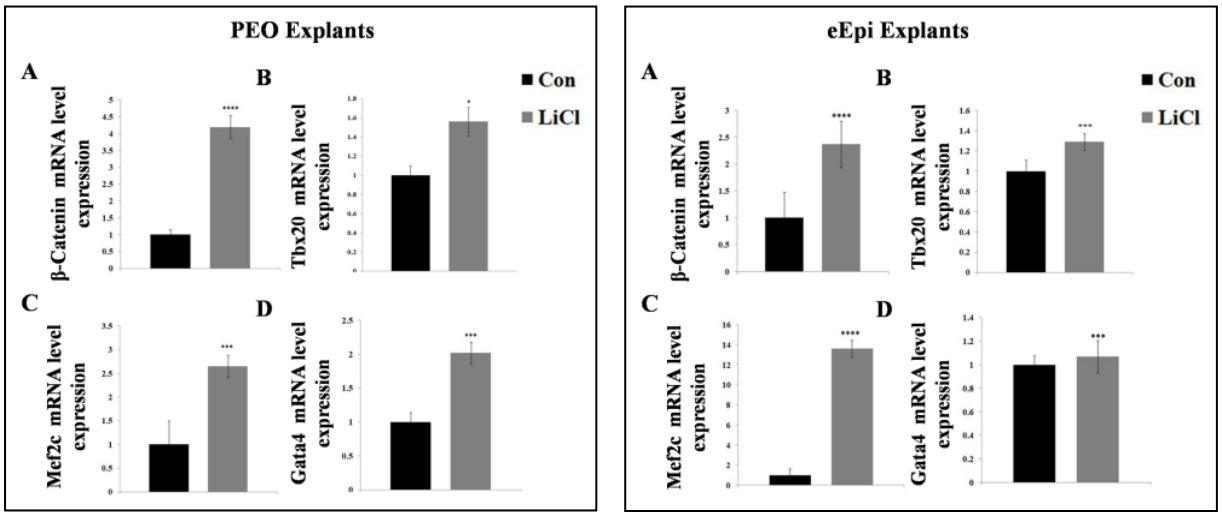
4.3. PEO derived cells show increased level of CM specific gene expression compared to eEpi cells



After successful establishment of both primary PEO and eEpi explant cultures, differential gene expression pattern of cardiomyocyte (CM) specific markers have been analyzed by semi qRT- PCR. CM specific markers (*Mef2c, Gata4*) have indicated increased band intensity in PEO compared to eEpi cells (figure A). *Gapdh* is used as loading control. These data suggest that specifically CM markers have increased expression in PEO compared to eEpi cells. Overall, these results might suggest CM specification capacity of both PEO and eEpi cells towards multiple cardiac lineage specification at least in culture.

4.4. LiCl mediated Wnt signaling manipulation in both PEO and eEpi cells show increased CM lineage specific gene expression

To induce Wnt/β-catenin signaling, avian primary PEO and eEpi explant cultures are treated with 15mM of LiCl. After that CM specific gene marker expressions have been analysed. Real time data demonstrates that the Wnt signaling specific *β-catenin* expression is significantly upregulated by 4.19 in PEO cells and 2.37 fold in eEpi cells upon LiCl treatment



of (figure A and E). Expressions of *Tbx20* (figure Band F) are also significantly upregulated by 1.56 fold in PEO Cells and 1.29 fold in eEpi cells after LiCl treatment. Also CM specific markers (*Mef2c, Gata4*) (figure C, D and G, H) have shown increased expressions by 2.51 fold and 2.02 fold in LiCl treatment of PEO cells. Similarly it has also increased expressions of 13.64 fold and 1.07 fold in LiCl treated eEpi cells. Thus, LiCl mediated *β-catenin* upregulation relates with increased CM differentiation potential of PEO and eEpi cells.

4.5. Increased level of β-catenin promotes increased number of cardiomyocyte in eEpi explant culture

Next, to validate the effect of Wnt signaling activation on CM differentiation, LiCl treated eEpi cells are immunostained with cytoplasmic sarcomeric myosin specific Mf20 antibody. Interestingly, increased numbers of Mf20 cells (arrowhead in

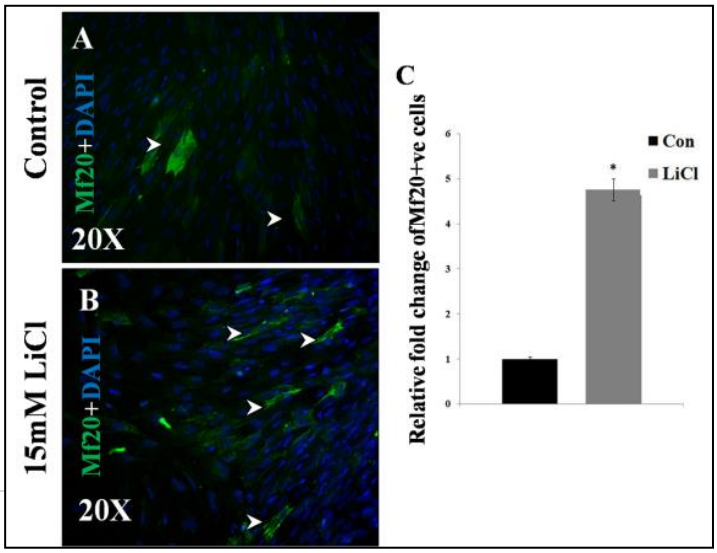
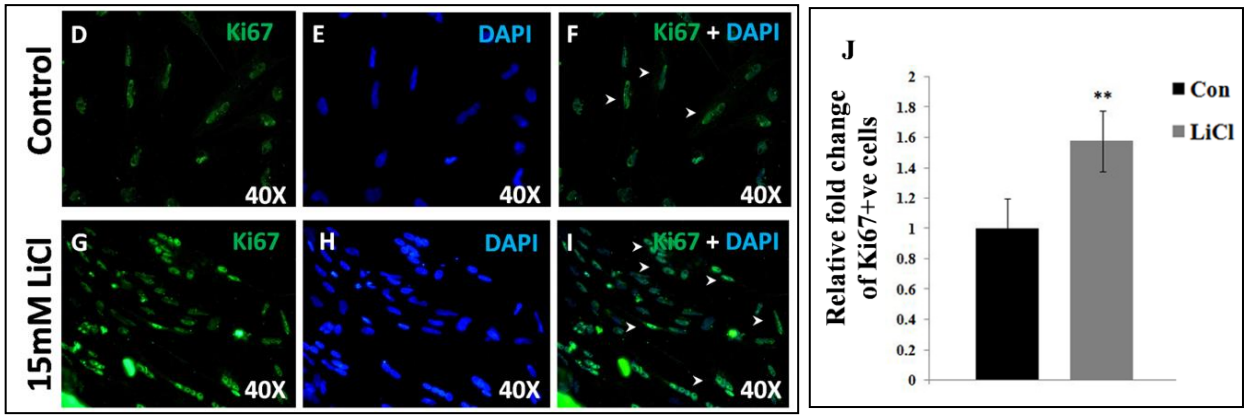


figure B) are detected compared to very few in untreated controls (arrowheads in figure A). Quantification of the data reveals 4.76 fold increase in Mf20 labeled cells in LiCl treated eEpi cells compared to untreated controls (figure C). Overall, LiCl mediated Wnt signaling activation promotes increased number of Mf20 positive cells in eEpi explant culture.

Proliferative indices are also detected in eEpi explant cultures with and without LiCl. Ki67 immunoreactivity is used to mark proliferating cells in eEpi explant cultures. Increased number of Ki67 positive nuclei are detected in LiCl treated eEpi cells (Figure G-I) compared to untreated controls (Figure D-F). Quantification of the data reveals around 1.58 fold

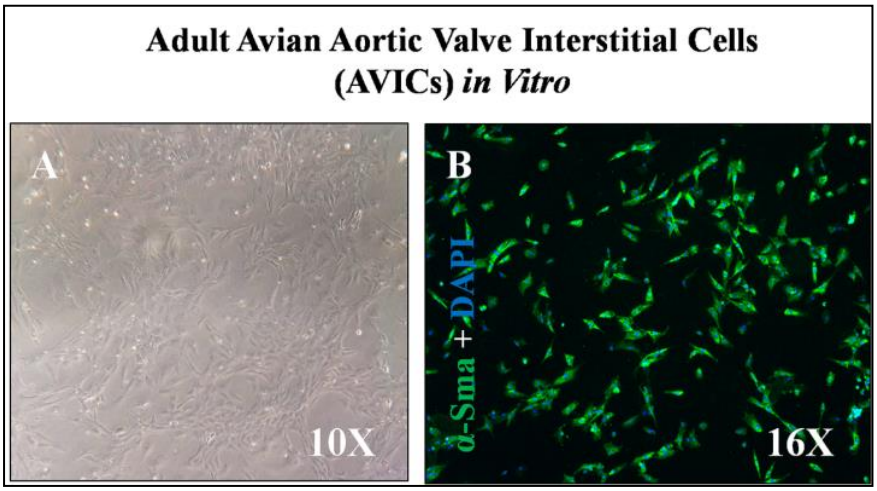


increased Ki67 immunoreactivity in treated eEpi cells (figure J). Overall these data suggest that LiCl mediated Wnt signaling activation via stabilization of β-catenin promotes generation of increased number of cycling CMs.

The following results are generated from proposed Aim 2

4.6. Establishment and characterization of Adult Avian Valve Interstitial Cells (AVICs) in culture with selective marker gene expression analyses

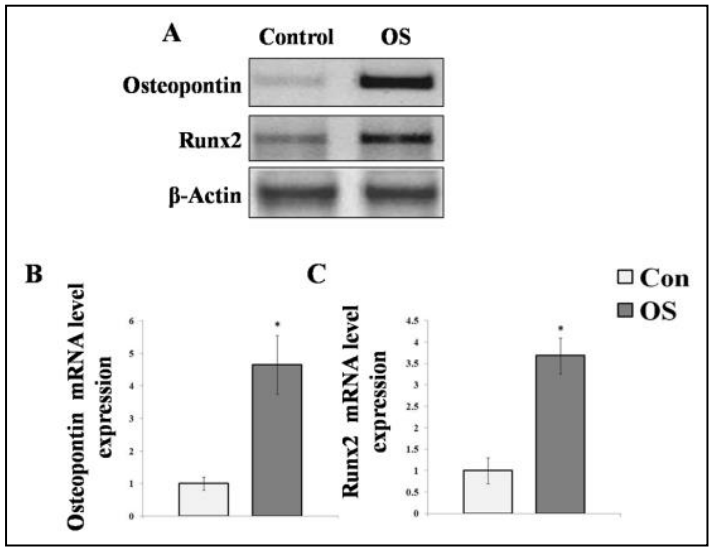
Avian adult AVICs have been cultured from isolated aortic tri-cusps from whole hearts. Firstly, whole hearts have been collected and three aortic valvular cusps are being dissected out from each sample. Following standardized protocol primary AVICs culture has been established successfully. Cells have started to grow and get clear filamentous AVIC morphology after 48-72hrs of seeding. Again bright field microscopic 10X image has been showing AVICs with well defined spindle shaped morphology (Figure A). To confirm about the phenotype of isolated adult AVICs, immunostaining was performed with a AVICs



marker, α-Sma which is shown in Figure B. Then after 4-5days of culture AVICs has been considered to perform further experiments. Hence, this data indicates successful establishment of adult avian valve interstitial cells (AVICs) in culture.

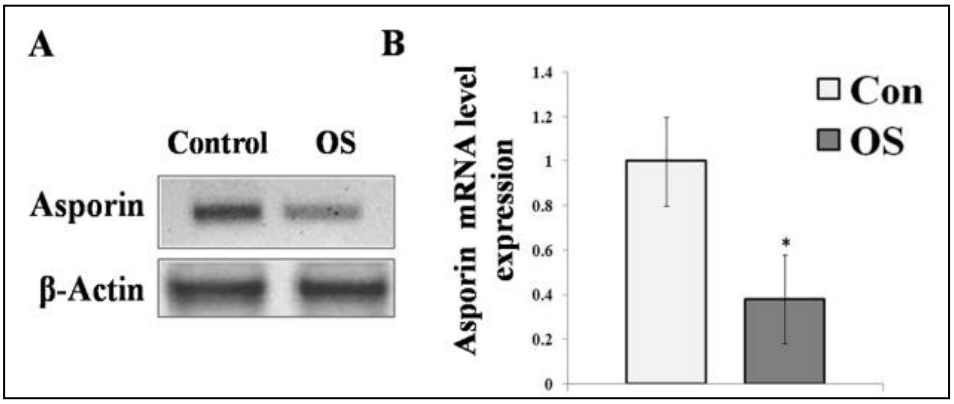
4.7. Osteogenesis induction on adult avian aortic valve interstitial cells (AVICs) in culture

After successful establishment of AVICs in culture and to induce osteogenesis, AVICs are treated with Osteogenic (OS) medium for 72hrs. OS medium was supplemented with OS ingredients [β-glycerophosphate (10mM), L-ascorbic acid (10nM), Dexomethasone (0.1μM)]. Semiq RT-PCR data indicates that the osteogenic signaling specific markers (*Osteopontin, Runx2)* show higher intense bands upon OS treatment on AVICs (Figure A). Likewise, in consistency with semi qRT-PCR data, *Osteopontin, Runx2* mRNA have shown increased expression (4.65, 3.68 folds respectively) by quantitative real time PCR (qRT-PCR) analyses in OS treated AVICs compared to untreated controls (Figure B & C). Overall, these data firmly suggest that upon osteogenic induction treating with OS medium resulted in increased expression of osteogenic lineage specific markers in AVICs. Thus, these data are evident for successful osteogenic induction on adult AVICs in culture.



4.8. *Asporin* expression analyses on cultured AVICs after OS induction

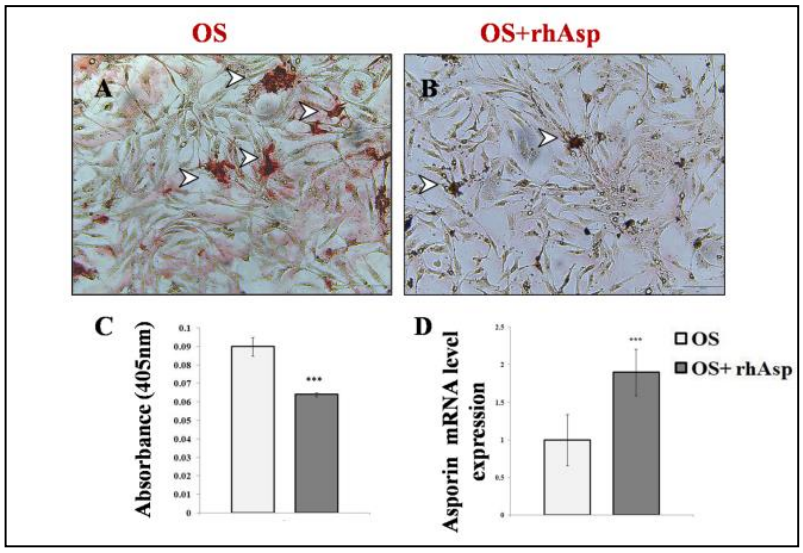
After successful establishment of adult AVICs cultures, gene expression of *Asporin* has been analyzed by semi qRT- PCR with 1µg of total RNA template, used to synthesize cDNA. At first, as expected, decreased expression of *Asporin* is determined on AVICs culture, performed by semi-qRT-PCR and also by qRT-PCR. In figure A, semi-qRT-PCR data indicates *Asporin*



expression is markedly downregulated in OS treated AVICs compared to control AVICs according to the band intensity. Moreover, in consistency with semi qRT-PCR data, *Asporin* mRNA have shown 0.38 fold decreased expression by qRT-PCR analyses in OS treated AVICs compared to untreated controls (figure B). So, these data suggests osteogenic induction of AVICs decreases *Asporin* expression at both mRNA and protein levels *in vitro* culture system.

4.9. Overexpression of Asporin decreases matrix mineralization on OS AVICs

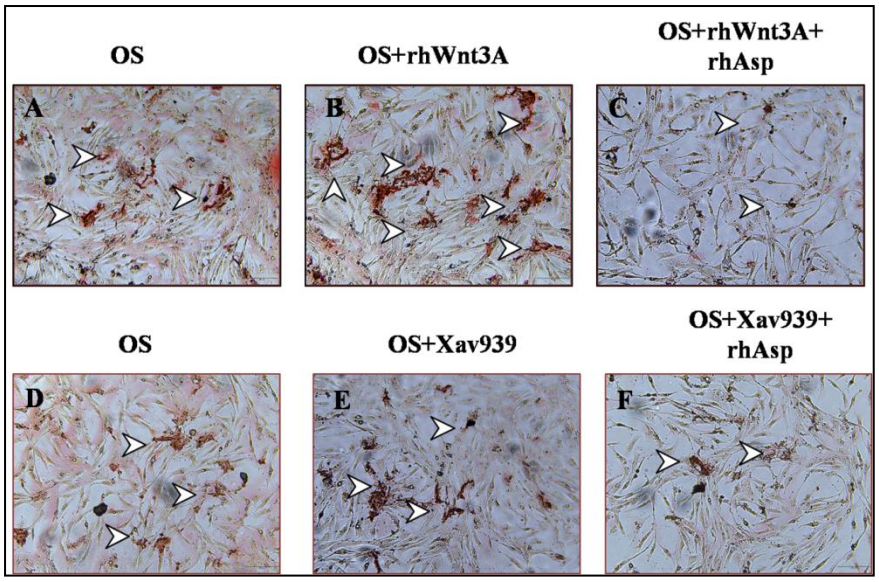
Next, to determine the effect of Asporin protein on AVICs mineralization, with OS induction cells were also treated with Asporin recombinant protein (rhAsp, 150ng/ml) for 24hrs to assess both qualitative and quantitative mineralization by Alizarin Red S staining. Also to confirm change in marker gene expression upon rhAsp treatment quantitative real time PCR (qRT-PCR) was performed. Figure A has shown Alizarin stained osteogenic AVICs (OS control) of total 6days of culture. Basal level of mineralization has been detected and marked



qualitatively (use small arrowheads in figures). Comparing with this OS control, reduced level of mineralization has been observed in figure B after treatment with rhASp treatment. Next, this data is further confirmed with alizarin quantitative assay in figure C. This quantitative data indicates decreased absorbance (OD) of alizarin stained rhAsp treated AVICs compared to OS control. After that, transcript level expression of *Asporin* gene is also evaluated on OS control and rhAsp treated OS AVICs by qRT-PCR (Figure D). These data detect that *Asporin* mRNA is significantly upregulated by 1.9 fold in rhAsp treated AVICs compared to OS control. Hence, above data have been stating that Asporin might involve to reduce valvular mineralization.

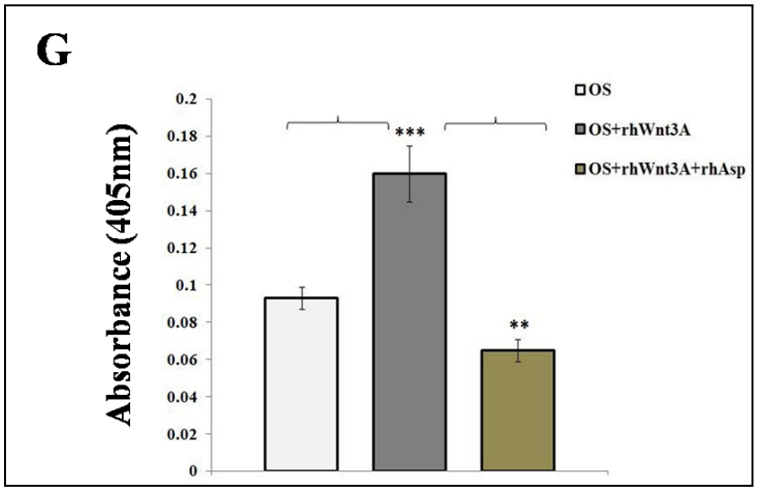
Together, these data strongly suggest that overexpression of Asporin diminishes the level mineralization in adult avian AVICs *in vitro*.

4.10. Asporin function in AVICs mineralization following Wnt signaling manipulation



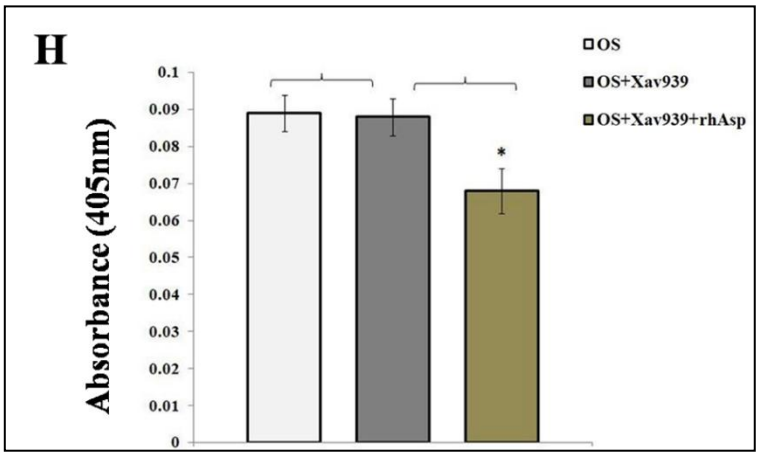
Next, to determine the functional relation of Wnt signaling with Asporin in AVICs mineralization, cells were further treated with Wnt3A recombinant protein (rhWnt3A, 150ng/ml, 24hrs) to activate Wnt signaling and also Asporin recombinant protein (rhAsp, 150ng/ml, 24hrs). Reversely, cells were treated with

Wnt signaling inhibitor Xav939 (2μM, for 24hrs) to inhibit Wnt signaling and also Asporin recombinant protein (rhAsp, 150ng/ml), for 24hrs in both cases. Then cells were used to assess qualitative and quantitative mineralization performing Alizarin Red S staining of osteogenic AVICs. Basal level of mineralization has been detected and marked qualitatively



in figure A & D. Comparing with this OS control, increased level of mineralization has been detected in rhWnt3a treated OS AVICs (Figure B). Interestingly, in figure C when OS AVICs were treated with both rhWnt3A and rhAsp then mineralization level, detected by Alizarin absorbance, has got reduced qualitatively. These data is further validated following alizarin quantitative assay in figure G. It also has shown that increased absorbance (OD, 0.067) of alizarin of in rhWnt3A treated AVICs compared to OS control. In addition, decreased absorbance (OD, 0.095) of alizarin has been detected when OS AVICs were treated with both rhWnt3A and rhAsp.

On the other hand, comparing with this OS control, level of mineralization has not been detected as significant change in Xav939 treated OS AVICs (Figure E). Interestingly, in figure F when OS AVICs were treated with both rhWnt3A and rhAsp then mineralization



level, detected by Alizarin absorbance, has got reduced qualitatively. These data is further validated following alizarin quantitative assay in figure H. Also it has shown increased absorbance (OD, 0.01) of alizarin in Xav939 treated AVICs compared to OS control. In addition, decreased absorbance (OD, 0.021) of alizarin has been detected when OS AVICs were treated with both Xav939 and rhAsp. Therefore, it is highly suggestive of the existence

of an Asporin dependent anti-mineralization and/or anti-calcific pathway sensitive to Wnt signaling induced AVICs with mineralization and/or calcification in vitro.

Together, all these data strongly depict that valvular mineralization/calcification mechanism might be dependent on Asporin mediated Wnt/β-catenin pathway on adult avian AVICs *in vitro*.

5. Conclusion and Impact:

This report has been aimed at two important cardiac research areas, namely **epicardial differentiation biology** and **aortic valve calcification**.

The first aim reveals that Proepicardial organ (PEO) derived cells have increased differentiation potential compared to embryonic epicardial (eEpi) cells for cardiomyocyte (CM) lineage differentiation and Wnt/β-catenin signaling plays an important role in the differentiation process. PEO-derived cells have shown increased CM specific marker gene expressions, including *Tbx20* compared to EPDCs. Wnt signaling manipulation also results in increased number of CM lineage specification in eEpi derived cells *in vitro*. Current research in cardiac regenerative biology emphasizes the search for the developmental cues shown to be reactivated or induced after adult cardiac injuries in order to repair and/or regenerate the injured heart. Therefore, search for such cardiac progenitor cells with increased potential for other cardiac lineage differentiation might provide us further impetus for better cell based repair and regenerative therapy in clinical settings.

Asporin, a small leucine rich proteoglycan negatively regulates bone cell differentiation, and also expressed in adult mouse aortic valves with unknown functional significance. The second aim of this report shows decreased level of *Asporin* mRNA in cultured adult AVICs with induced mineralization and concomitant increased level of Wnt/β-catenin signaling. In contrast and as hypothesized, overexpression of Asporin significantly decreases AVICs matrix mineralization associated with reduced levels of Wnt/β-catenin signaling.Therefore, in human heart valve disease, the search for natural molecules with anti-calcific properties like Asporin and its associated regulatory signaling might provide us future implications for better drug based clinical treatments against aortic valve mineralization and calcification.

6. List of Abbreviations:

MI: Myocardial Infarction

CM: Cardiomyocyte

PEO: Proepicardial Organ

AV: Atrio-ventricular

EPDC: Epicardium derived cell

EMT: Epithelial to meshenchymal transition

MSC: Mechenchymal cell

OFT: Out-flow-tract

ECM: Extracellular matrix

CAVD: Calcific Aortic Valve Diseases

PLAP1: Periodontal ligament associated protein 1

AVIC: Aortic valvular interstitial cell

eEpi: Embryonic Epicardial

OS: Osteogenic

RT-PCR: Reverse Transcriptase PCR

qRT-PCR: Quantitative Real Time PCR

IF: Immunofluorescence

dH_2O: Distilled Water

LiCl: Lithium Chloride

rh: Recombinant Human

GOF: Gain of function

7. Table I: Designed primer lists (IDT primer quest tool)

Genes	Annealing temperature, (°C)	Product Size (Base Pair)	Primer sequences
Gallus gallus			
Tbx20	56.1	276	F:5′- CAGGCAACGCAAAGCAGAG-3′ R:5′-TTGGCATGTGGAAAGAAGG-3’
Gata4	59.2	360	F:5'-GTGTCACCTCGCTTCTCCTT-3' R:5'-GTGCCCTGTGCCATCTCT-3'
Mef2c	56.1	304	F:5'-GAT TTC CAC TCT CCT GT-3' R:5'-TTA CCA TGG GAC ATC T-3'
Wt1	60	170	F:5'-AAAACGCCCCTTCATGTGTG-3' R:5'-GTCTTTGGTGCCGTTTCAGT-3'
Tbx18	53.8	177	F:5’-TAATGCTGACTCCCCGGTAC-3’ R:5’-TGGACACGAGGCTGGTATTT-3’
Asporin	51	328	F:5'-GCAACATTCCACCAGATAC-3' R:5'-AGAGGATTTGCACTCATTTC-3'
Osteopontin	59.2	198	F:5′-GCAGCAGACACAGAATGACC-3′ R:5′-TCTGTGGGGAAGTCTGTGAC-3′
Runx2	60.9	211	F:5'-CCCCGGCTCCTCCCAAAACCAA-3' R:5'-CCGCCTCCACACCGTCACTCTG-3'

β-catenin	56.1	205	F:5'ACTGTTCTACGCCATCACCA-3' R:5'-ACCACTGGCCAGAATGATGA- 3'
Gapdh	56.1	176	F:5'- GGCATTGCACTGAATGACCAT-3' R:5'-TCTCCCACCTCCCCCAGGTG- 3'
β-actin	55	139	F:5'-AGTACCCCATTGAACACGGT-3' R:5'-ATACATGGCTGGGGTGTTGA-3'

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